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Role of mitochondrial inner membrane organizing system in protein biogenesis of the mitochondrial outer membrane

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ABSTRACT **Mitochondria contain two membranes, the outer membrane and the inner membrane with folded cristae. The mitochondrial inner membrane organizing system (MINOS) is a large protein complex required for maintaining inner membrane architecture. MINOS interacts with both preprotein transport machineries of the outer membrane, the translocase of the outer membrane (TOM) and the sorting and assembly machinery (SAM). It is unknown, however, whether MINOS plays a role in the biogenesis of outer membrane proteins. We have dissected the interaction of MINOS with TOM and SAM and report that MINOS binds to both translocases independently. MINOS binds to the SAM complex via the conserved polypeptide transport–associated domain of Sam50. Mitochondria lacking mitofilin, the large core subunit of MINOS, are impaired in the biogenesis of β-barrel proteins of the outer membrane, whereas mutant mitochondria lacking any of the other five MINOS subunits import β-barrel proteins in a manner similar to wild-type mitochondria. We show that mitofilin is required at an early stage of β-barrel biogenesis that includes the initial translocation through the TOM complex. We conclude that MINOS interacts with TOM and SAM independently and that the core subunit mitofilin is involved in biogenesis of outer membrane β-barrel proteins.**

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Abbreviations used: $DiSC_3(5)$, dipropylthiadicarbocyanine iodide; ERMIONE, endoplasmic reticulum–mitochondria organizing network; Fcj1, formation of crista junction protein 1 (mitofilin); IgG, immunoglobulin G; MIA, mitochondrial intermembrane space assembly; MINOS, mitochondrial inner membrane organizing system; PMSF, phenylmethylsulfonyl fluoride; POTRA, polypeptide transport-associated; SAM, sorting and assembly machinery; TEV, tobacco etch virus; TOM, translocase of outer mitochondrial membrane.

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INTRODUCTION

Mitochondria consist of two membranes and two aqueous compartments, intermembrane space and matrix. The inner membrane is folded into tubular invaginations called cristae. Cristae junctions connect the cristae membranes with the remainder of the inner membrane, which is adjacent to the outer membrane and is called the inner boundary membrane (Frey and Mannella 2000; Mannella, 2006; Zick *et al.*, 2009). Recent studies led to the identification of a large protein complex of the inner membrane that plays a crucial role in the maintenance of inner membrane architecture. The complex was termed mitochondrial inner membrane organizing system (MINOS), mitochondrial contact site complex, or mitochondrial organizing structure (Harner *et al.*, 2011; Hoppins *et al.*, 2011; Herrmann, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; van der Laan *et al.*, 2012). MINOS consists of six subunits that are all inner membrane proteins exposed to the intermembrane space. Two core proteins, mitofilin (formation of crista junction protein 1 [Fcj1]) and

Mio10 (Mcs10/Mos1/MINOS1), are essential for keeping the cristae membranes attached to the inner boundary membrane (John *et al.*, 2005; Rabl *et al.*, 2009; Mun *et al.*, 2010; Harner *et al.*, 2011; Head *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; Zerbes *et al.*, 2012). The additional subunits, Aim5 (Mcs12), Aim13 (Mcs19/MINOS3), Aim37 (Mcs27), and Mio27 (Mcs29/Mos2), contribute to the integrity of the MINOS complex and maintenance of cristae architecture.

In addition to its role in inner membrane architecture, MINOS was found to interact with protein complexes of the outer mitochondrial membrane, including the two essential preprotein transport machineries (Xie *et al.*, 2007; Darshi *et al.*, 2011; Harner *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; Körner *et al.*, 2012; Ott *et al.*, 2012; Zerbes *et al.*, 2012). The translocase of the outer mitochondrial membrane (TOM) forms the main entry gate for most nuclear-encoded mitochondrial precursor proteins, whereas the sorting and assembly machinery (SAM) mediates the insertion of β-barrel proteins into the mitochondrial outer membrane (Dolezal *et al.*, 2006; Neupert and Herrmann, 2007; Chacinska *et al.*, 2009; Dukanovic and Rapaport, 2011; Endo *et al.*, 2011; Becker *et al.*, 2012). These contact sites between inner and outer membranes are involved in the maintenance of cristae morphology (Körner *et al.*, 2012; Ott *et al.*, 2012). In addition, it was shown that mitofilin/Fcj1 supports the transport of small precursor proteins into the intermembrane space (von der Malsburg *et al.*, 2011). Mitofilin contains a large intermembrane space domain that interacts with the TOM complex and the receptor Mia40 of the mitochondrial intermembrane space assembly (MIA) machinery (Chacinska *et al.*, 2004; Mesecke *et al.*, 2005; Dabir *et al.*, 2007; Grumbt *et al.*, 2007; Stojanovski *et al.*, 2008; Banci *et al.*, 2009; Kawano *et al.*, 2009; Koehler and Tienson, 2009; Bien *et al.*, 2010). The transient interaction of mitofilin with Mia40 helps to position this intermembrane space receptor in the vicinity of the TOM complex, and precursor proteins passing through the TOM channel can therefore be immediately captured by Mia40 (von der Malsburg *et al.*, 2011).

The mitochondrial outer membrane contains two major protein types: proteins with α-helical transmembrane segments and β-barrel proteins. The precursors of β-barrel proteins are initially imported via the TOM complex and translocated to the intermembrane space (Model *et al.*, 2001; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003, 2004; Mihara, 2003). Chaperone complexes of the small TIM-type help in transfer of the hydrophobic precursors to the SAM complex that mediates insertion of the proteins into the outer membrane (Paschen *et al.*, 2003, 2005; Wiedemann *et al.*, 2003, 2004; Gentle *et al.*, 2004; Hoppins and Nargang, 2004; Habib *et al.*, 2005; Chan and Lithgow, 2008; Kutik *et al.*, 2008). For α-helical precursor proteins, several import pathways have been described that can involve TOM receptors, SAM, and other outer membrane proteins (Stojanovski *et al.*, 2007a; Becker *et al.*, 2008, 2009, 2011; Hulett *et al.*, 2008; Kemper *et al.*, 2008; Popov-Čeleketić *et al.*, 2008; Thornton *et al.*, 2010; Dukanovic and Rapaport, 2011; Papić *et al.*, 2011; Dimmer *et al.*, 2012). Because MINOS interacts with both outer membrane translocases, it may potentially be connected to protein import into the outer membrane. However, different views on the relation of MINOS to the biogenesis of outer membrane proteins have been reported (Darshi *et al.*, 2011; Körner *et al.*, 2012).

For this report, we analyzed the interaction of MINOS with TOM and SAM. We show that MINOS binds to TOM and SAM in an independent manner. Mutant mitochondria lacking mitofilin/Fcj1 were impaired in the biogenesis of β-barrel proteins. Mitofilin is involved in an early stage of β-barrel import that includes the translocation of precursor proteins through the TOM complex. We conclude that mitofilin not only promotes the import of small proteins into the intermembrane space (von der Malsburg *et al.*, 2011) but also the biogenesis of β-barrel proteins of the outer membrane.

RESULTS

Differential copurification of TOM and SAM with MINOS subunits

We asked whether MINOS interacts with TOM and SAM simultaneously or whether distinct outer membrane contacts exist. In a first approach, we performed pulldown experiments with Protein A–tagged MINOS components. We used a *Saccharomyces cerevisiae* strain expressing mitofilin/Fcj1 with a C-terminal protein A tag (von der Malsburg *et al.*, 2011) and generated a yeast strain that expressed Mio27 with a C-terminal protein A tag. The protein A tags were attached to the MINOS components via a linker containing a tobacco etch virus (TEV) protease cleavage site. Extracts of the yeast cells were prepared using the nonionic detergent digitonin and subjected to immunoglobulin G (IgG) affinity chromatography. Bound proteins were eluted by cleavage with TEV protease. $Fcj1_{ProtA}$ and $Mio27_{ProtA}$ copurified the other five MINOS subunits with comparable efficiency (Figure 1, lanes 5 and 6), whereas control proteins of the inner and outer membranes were not found in the eluate (Figure 1, lanes 5, 6, 11, and 12). In addition to the MINOS subunits, $Fcj1_{ProtA}$ copurified TOM and SAM subunits (Figure 1, lane 11; the receptor Tom70 is only loosely associated with the yeast TOM complex and is therefore copurified in minor amounts; Meisinger *et al.*, 2001). In contrast, $Mio27_{ProtA}$ neither copurified the channel-forming protein Tom40 nor the receptors Tom20, Tom22, and Tom70

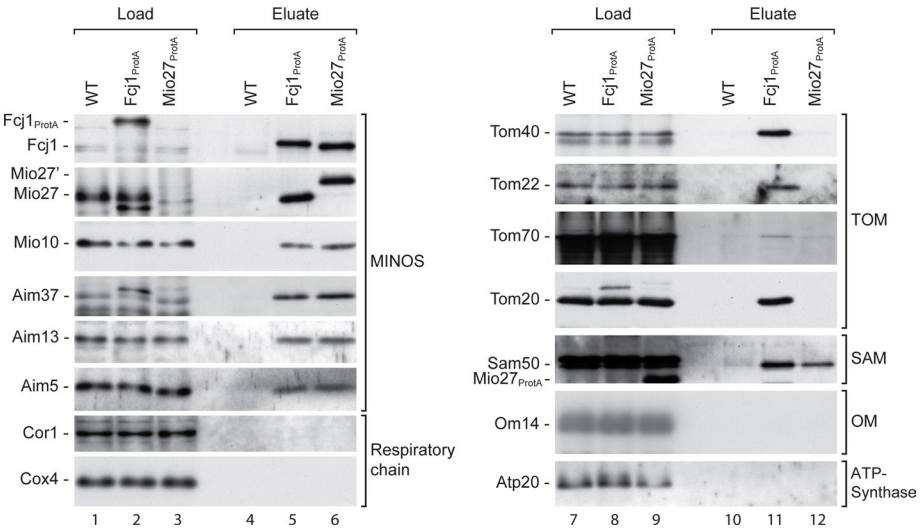

FIGURE 1: MINOS interacts with outer membrane protein complexes. Whole-cell digitonin extracts from wild-type (WT) cells and cells expressing protein A fusion constructs ($Fcj1_{ProtA}$ and $Mio27_{ProtA}$) were subjected to IgG affinity chromatography, elution with TEV protease, and analysis by SDS–PAGE and immunoblotting. Load, 1.5%; elution, 100%. Mio27′, TEV-cleaved form of $Mio27_{ProtA}$; OM, outer mitochondrial membrane.

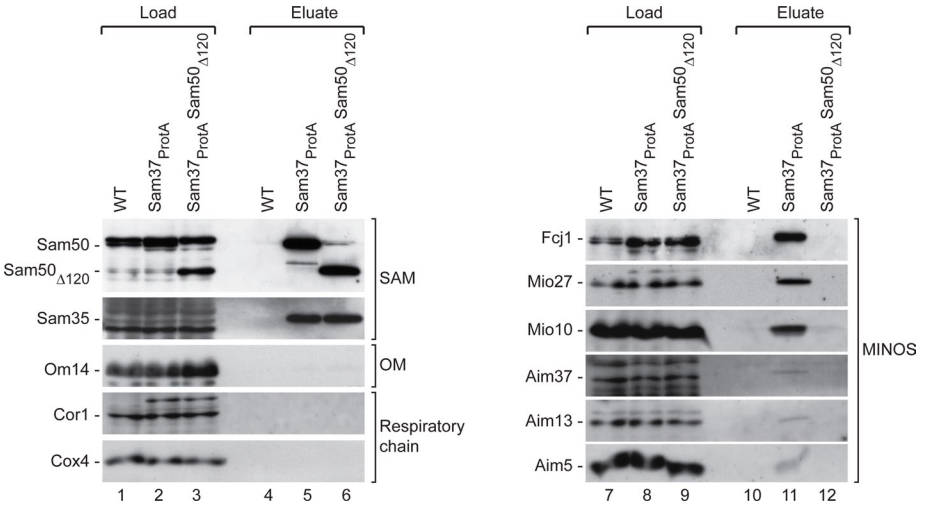

FIGURE 2: Interaction between MINOS and SAM depends on the POTRA domain of Sam50. Whole-cell powder from wild-type (WT), $Sam37_{ProtA}$, and $Sam37_{ProtA}$ $Sam50\Delta_{120}$ cells was solubilized in digitonin-containing buffer, subjected to IgG affinity chromatography, and analyzed by SDS–PAGE and immunoblotting. Load, 1%; elution, 100%. OM, outer mitochondrial membrane.

(Figure 1, lane 12), demonstrating that the TOM complex was not pulled down with tagged Mio27. However, $Mio27_{ProtA}$ copurified Sam50 (Tob55), the core component of the SAM complex, though with a reduced efficiency compared with the copurification with $Fcj1_{ProtA}$ (Figure 1, lanes 11 and 12). These findings raised the possibility that binding of TOM and SAM to MINOS is not coupled but can be separated.

Requirement of the polypeptide transport-associated domain of Sam50 for MINOS–SAM interaction but not for MINOS–TOM interaction

To obtain direct evidence for an independent interaction of MINOS with the outer membrane translocases, we sought determinants that are required for formation of the interactions. The SAM complex contains one large hydrophilic domain that is exposed to the intermembrane space, the polypeptide transport-associated (POTRA) domain at the N-terminus of Sam50 (Kozjak *et al.*, 2003; Paschen *et al.*, 2003; Sánchez-Pulido *et al.*, 2003; Gentle *et al.*, 2004; Habib *et al.*, 2007; Knowles *et al.*, 2008; Kutik *et al.*, 2008; Stroud *et al.*, 2011a), whereas Sam35 and Sam37 expose domains to the cytosolic side (Wiedemann *et al.*, 2003; Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004; Chan and Lithgow, 2008; Kutik *et al.*, 2008). We used a yeast strain that expressed Sam37 with a protein A tag (Kozjak *et al.*, 2003). From a digitonin extract of the cells, tagged Sam37 pulled down additional SAM proteins, Sam35 and Sam50, as expected (Figure 2, lane 5; Kozjak *et al.*, 2003), but also the subunits of MINOS (Figure 2, lane 11; Aim5, Aim13, and Aim37 were copurified with lower yield than Fcj1, Mio10, and Mio27). Control proteins of the outer and inner membranes were not copurified (Figure 2, lane 5). We generated a $Sam37_{ProtA}$ strain, in which the N-terminal 120 residues of Sam50, including the entire POTRA domain, were deleted ($Sam50_{\Delta120}$; Kutik *et al.*, 2008; Stroud *et al.*, 2011a). Copurification of the subunits of the SAM complex was not affected by the lack of the POTRA domain (Figure 2, lane 6; Habib *et al.*, 2007; Stroud *et al.*, 2011a). However, the pulldown of MINOS subunits with tagged Sam37 was strongly inhibited when the POTRA domain of Sam50 was lacking (Figure 2, lane 12). These results indicate that the POTRA domain is required for the interaction of the SAM complex with MINOS.

To address whether the POTRA domain was required for the interaction of TOM with MINOS, we generated an $Fcj1_{ProtA}$ strain in which the POTRA domain of Sam50 was deleted (Figure 3). Tagged Fcj1 pulled down the other five MINOS subunits independently of the presence or absence of the POTRA domain (Figure 3, lanes 5 and 6). The interaction of Fcj1 with Sam50 and Sam35 was strongly inhibited by the lack of the POTRA domain (Figure 3, lane 12; the steady-state levels of Sam50 and Sam35 were not affected; Figure 3, lanes 8 and 9). However, Tom22 and Tom40 were efficiently copurified with tagged Fcj1 and did not require the presence of the Sam50 POTRA domain (Figure 3, lanes 11 and 12). The mitochondrial ultrastructure analyzed by electron microscopy was not altered when mitofilin/Fcj1 carried a protein A tag and was only mildly affected by the lack of the Sam50 POTRA domain (Supplemental Figure S1), indicating that the POTRA-mediated MINOS–SAM interaction is not strictly essential for maintaining the architecture of the mitochondrial inner membrane.

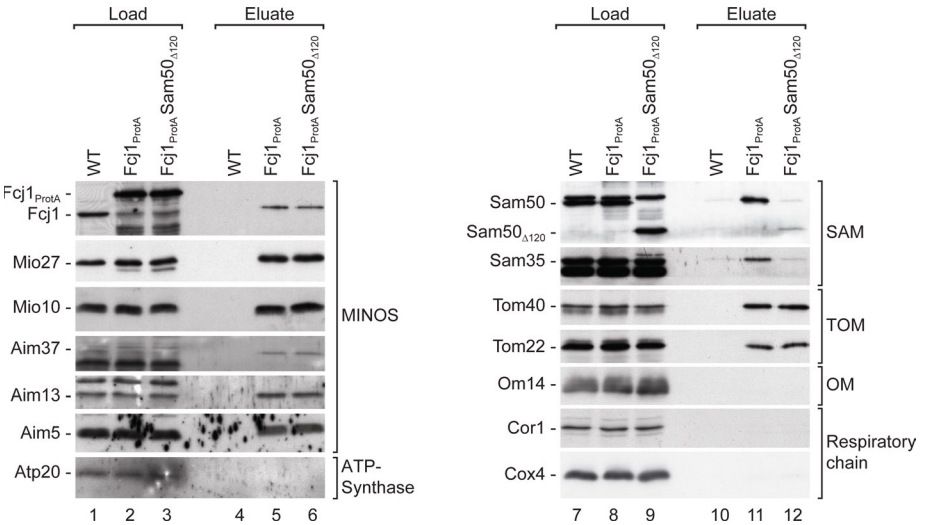

FIGURE 3: MINOS independently interacts with outer membrane TOM and SAM complexes. Whole-cell digitonin extracts from wild-type (WT), $Fcj1_{ProtA}$, and $Fcj1_{ProtA}$ $Sam50\Delta_{120}$ cells were subjected to IgG affinity chromatography and analyzed by SDS–PAGE and immunoblotting. Load, 1.5%; elution, 100%. OM, outer mitochondrial membrane.

We conclude that mitofilin/Fcj1 can bind TOM despite an impaired interaction between mitofilin/Fcj1 and SAM. In Figure 1 we show that tagged Mio27 pulled down

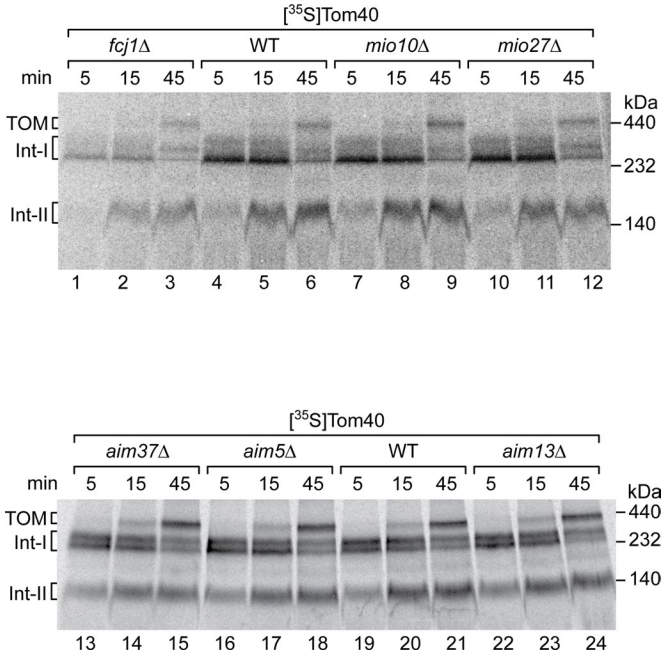

FIGURE 4: Deletion of *FCJ1*, but not of other MINOS components, leads to impaired Tom40 biogenesis. [35S]-labeled Tom40 was imported into mitochondria isolated from wild-type (WT), *fcj1*Δ, *mio10*Δ, *mio27*Δ, *aim37*Δ, *aim5*Δ, and *aim13*Δ cells. Mitochondria were solubilized with digitonin and subjected to blue native electrophoresis and digital autoradiography. Int-I, precursor-SAM assembly intermediate I; Int-II, assembly intermediate II.

SAM but not the TOM complex, indicating that the MINOS–SAM interaction does not require interaction with the TOM complex. Taken together, these findings demonstrate that TOM and SAM independently interact with components of the MINOS machinery of the inner membrane.

Biogenesis of outer membrane proteins in MINOS mutant mitochondria

Biogenesis of mitochondrial β-barrel proteins requires both TOM and SAM (Endo and Yamano, 2009; Dukanovic and Rapaport, 2011; Becker *et al.*, 2012). To study whether MINOS was involved in outer membrane protein biogenesis, we used the radiolabeled precursor of Tom40 as a model substrate. For this precursor, three assembly stages can be resolved by blue native electrophoresis of digitonin-lysed mitochondria (Model *et al.*, 2001; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003; Ishikawa *et al.*, 2004; Chan and Lithgow, 2008; Dukanovic *et al.*, 2009). On incubation with isolated mitochondria, Tom40 forms intermediate I, which represents interaction of the precursor with the SAM complex (Model *et al.*, 2001; Wiedemann *et al.*, 2003; Becker *et al.*, 2010). Subsequently, the precursor forms a smaller intermediate II before it is assembled into the mature TOM complex (Figure 4, lanes 4–6). We isolated mitochondria from single-deletion yeast strains of the six MINOS subunits and imported the precursor of Tom40. Most of the mutant mitochondria imported and assembled Tom40 in a manner similar to wild-type mitochondria (Figure 4, lanes 4–24). The only exception was mitochondria lacking mitofilin/Fcj1, which were considerably impaired in all three assembly stages of Tom40 (Figure 4, lanes 1–3).

We analyzed the steady-state levels of TOM, SAM, and TIM proteins and did not observe any substantial differences between *fcj1*Δ and wild-type mitochondria that would explain the defect in Tom40 biogenesis (Figure S2A). Moreover, the stability of neither the TOM complex nor the SAM complex, as analyzed by blue native electrophoresis, was affected by the lack of mitofilin/Fcj1 (Figure S2B). The biogenesis of other β-barrel proteins of the outer mitochondrial membrane, porin and Mdm10, was analyzed by monitoring assembly of the radiolabeled precursors. These precursors are also imported via the TOM and SAM complexes but with faster kinetics than Tom40, and the wild-type precursors therefore do not form stable SAM intermediates in considerable amounts (Wiedemann *et al.*, 2003; Kutik *et al.*, 2008; Stroud *et al.*, 2011a). Biogenesis of porin and Mdm10 was retarded in *fcj1*Δ mitochondria but not in *mio10*Δ mitochondria (Figure S2, C and D), supporting the view that mitofilin/Fcj1, but not Mio10, is involved in the efficient import of outer membrane β-barrel proteins.

We performed several control experiments to exclude possible indirect effects. 1) Major alterations of mitochondrial phospholipid levels, such as lack of cardiolipin, can impair the biogenesis of outer membrane proteins (Gebert *et al.*, 2009). We compared the phospholipid composition of mitochondria from *fcj1*Δ and wild-type yeast. In addition, mitochondria lacking Mio10 were analyzed. Figure S3 shows that the phospholipid composition was not substantially changed by the lack of the central MINOS components mitofilin/Fcj1 or Mio10. 2) We generated a yeast strain in which *FCJ1* was expressed under the control of a galactose-inducible promoter. On shift of the cells to glucose-containing medium, the levels of Fcj1 were decreased (the levels of TOM, SAM, and TIM components, as well as the stability of TOM and SAM complexes, were not affected; Figure S4, A and B). We selected conditions under which Fcj1 was strongly depleted (Figure S4A), yet the inner membrane potential $\Delta\psi$ was comparable with that of wild-type mitochondria (Figure 5A). For *fcj1*Δ mitochondria, it has been reported that $\Delta\psi$ is partially decreased, and the $\Delta\psi$-dependent import of proteins into or across the inner membrane is therefore also partially reduced (von der Malsburg *et al.*, 2011). In contrast, the Fcj1-depleted mitochondria imported the matrix protein F_1-ATPase subunit β and the inner membrane proteins cytochrome c_1 and ADP/ATP carrier with an efficiency close to that of wild-type mitochondria (Figure 5, B and C). Though the import of mitochondrial outer membrane proteins does not require the inner membrane potential (Chacinska *et al.*, 2009), use of the Fcj1-depleted mitochondria offered the opportunity to minimize pleiotropic effects. The assembly of the precursor of Tom40 was impaired in Fcj1-depleted mitochondria (Figure 5D), supporting the view of a specific role of mitofilin/Fcj1 in this process.

We conclude that lack of mitofilin/Fcj1 impairs the biogenesis pathway of β-barrel precursors, whereas other MINOS components are not required for β-barrel assembly.

Lack of mitofilin/Fcj1 impairs biogenesis of Tom40 at a stage before the SAM complex

At which stage of Tom40 assembly is mitofilin/Fcj1 involved? Because the formation of the SAM-bound state (assembly intermediate I on native gels) is strongly impaired in *fcj1*Δ mitochondria, either the SAM complex itself or a step leading to the SAM complex is compromised. We imported the radiolabeled precursor of Tom22 that uses TOM receptors and each subunit of the SAM complex before its assembly into the TOM complex (Keil and Pfanner, 1993; Meisinger *et al.*, 2004; Stojanovski *et al.*, 2007a; Dukanovic *et al.*, 2009; Thornton *et al.*, 2010). Assembly of radiolabeled Tom22 into the TOM complex occurred with similar efficiency in *fcj1*Δ mitochondria, *mio10*Δ mitochondria, and wild-type mitochondria (Figure S5A). Thus the SAM complex and TOM receptors are functional in

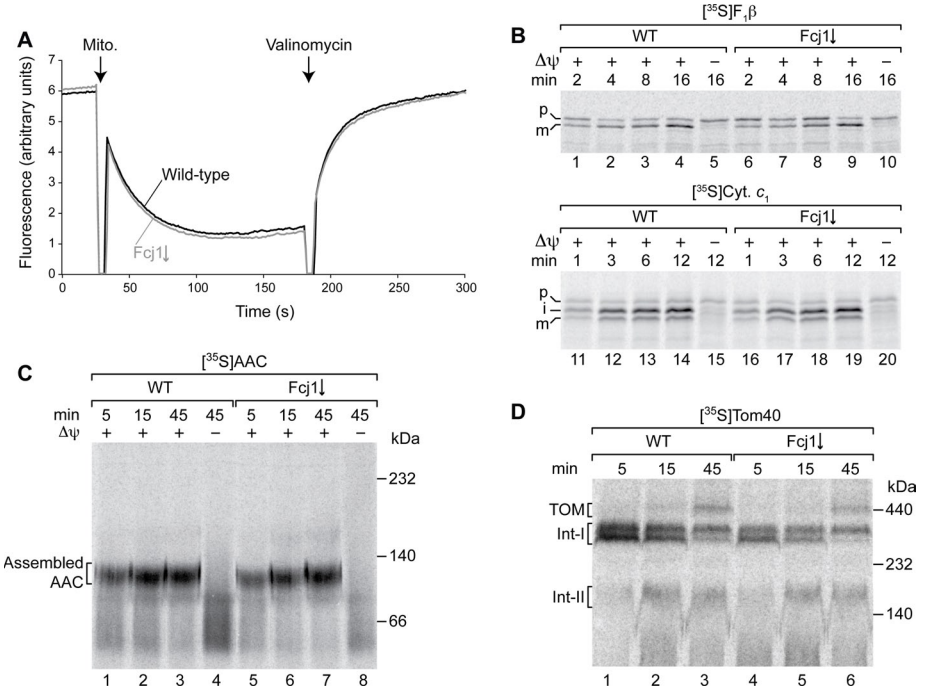

FIGURE 5: Biogenesis of Tom40 is impaired upon depletion of Fcj1. (A) Fcj1↓ (YPH499 *fcj1::kanMX6, PGAL1-FCJ1*) and wild-type control cells were precultured in the presence of 2% galactose. Subsequently, expression of *PGAL1-FCJ1* was inhibited with 1% glucose, mitochondria were isolated, and the mitochondrial membrane potential was assessed using the potential-sensitive dye $DiSC_3(5)$. (B) The [^{35}S]-labeled precursors of F_1-ATPase subunit β ($F_1β$) and cytochrome c_1 (Cyt. c_1) were imported into isolated mitochondria for the indicated time periods. After proteinase K treatment to remove nonimported precursors, samples were analyzed by SDS–PAGE and digital autoradiography. p, precursor; i, intermediate; m, mature. (C) [^{35}S]ADP/ATP carrier (AAC) or (D) [^{35}S]Tom40 was imported into isolated mitochondria as indicated and analyzed by blue native electrophoresis and digital autoradiography. Int-I, precursor-SAM assembly intermediate I; Int-II, assembly intermediate II.

*fcj1*Δ mitochondria (the precursor of Tom22 is not translocated through the TOM channel to the intermembrane space but is directly inserted into the outer membrane by the SAM complex; Stojanovski *et al.*, 2007a; Thornton *et al.*, 2010). Import and assembly of the precursor of Tom5 into the TOM complex was also not affected by the lack of Fcj1 or Mio10 (Figure S5B).

Because the POTRA domain is required for the MINOS–SAM interaction, we compared Tom40 assembly in $Sam50_{Δ120}$ mitochondria with Tom40 assembly in *fcj1*Δ mitochondria. It has been reported that lack of the POTRA domain only mildly affects the biogenesis of radiochemical amounts of Tom40 (Kutik *et al.*, 2008; Stroud *et al.*, 2011a), and *fcj1*Δ mitochondria therefore apparently show a much stronger defect in Tom40 biogenesis. To directly compare this with the pulldown experiments that were dependent on the presence of the POTRA domain (Figure 2), we studied the import of Tom40 into $Sam37_{ProtA}$ mitochondria lacking the POTRA domain of Sam50. Tom40 assembly was not inhibited in the POTRA-deficient mutant mitochondria (Figure 6A). Because the POTRA domain is required for a stable MINOS–SAM interaction, the biogenesis pathway of Tom40 is not inhibited when the MINOS–SAM interaction is disturbed. Taken together with the full activity of the SAM complex in the assembly of Tom22, these results suggest that the Tom40 assembly defect in *fcj1*Δ mitochondria may occur at a step preceding formation of the SAM-precursor intermediate.

The early steps in Tom40 biogenesis involve initial translocation across the outer membrane to the intermembrane space and binding to the small TIM chaperones (Model *et al.*, 2001; Mihara, 2003; Wiedemann *et al.*, 2003, 2004; Hoppins and Nargang, 2004). These early steps do not involve blue native–stable intermediates and thus cannot be directly visualized by native gel electrophoresis (Wiedemann *et al.*, 2004; Rao *et al.*, 2012). After a short import time, the precursor of Tom40 was associated with tagged Fcj1 (Figure S5C), suggesting an involvement of mitofilin/Fcj1 at an early import stage. The translocation of the Tom40 precursor across the outer membrane can be assessed by its protection against externally added protease (Wiedemann *et al.*, 2003, 2004; Paschen *et al.*, 2003; Chan and Lithgow, 2008; Dukanovic *et al.*, 2009). We imported radiolabeled Tom40 precursor into isolated mitochondria. The efficiency of Tom40 translocation to a protease-protected location was significantly reduced in *fcj1*Δ mitochondria compared with wild-type mitochondria (Figure 6, B and C), indicating that this initial import step of Tom40 is affected by the lack of mitofilin/Fcj1.

Taken together, mitochondria lacking mitofilin/Fcj1 are impaired in an early step of Tom40 biogenesis that precedes the SAM complex and includes the initial translocation across the outer membrane.

DISCUSSION

We report a new function for mitofilin/Fcj1 and the MINOS machinery of the mitochondrial inner membrane. This membrane organizing system is not only involved in the maintenance of mitochondrial cristae morphology (Harner *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; Körner *et al.*, 2012; Ott *et al.*, 2012; Zerbes *et al.*, 2012) and protein import via the MIA pathway into the intermembrane space (von der Malsburg *et al.*, 2011), but also in the biogenesis of outer membrane proteins with β-barrel topology.

Both protein translocases of the outer membrane, TOM and SAM, independently bind to MINOS. 1) We observed for the SAM complex that the conserved POTRA domain on the intermembrane space side of Sam50 is required for the stable interaction with MINOS, whereas the binding of TOM to MINOS occurs independently of the POTRA domain. 2) The TOM complex is efficiently pulled down only by tagged mitofilin/Fcj1 but not by other tagged subunits of MINOS, such as Mio27 and Aim5, although these components copurify all other MINOS subunits (von der Malsburg *et al.*, 2011; this study). In contrast, the SAM complex is not only copurified with mitofilin/Fcj1 but also with several other MINOS components (Xie *et al.*, 2007; Darshi *et al.*, 2011; Harner *et al.*, 2011; Alkhaja *et al.*, 2012; this study). Thus MINOS can be found in association with the SAM complex independently of the MINOS–TOM interaction. 3) When the MINOS complex is fully or partially disrupted by deletion of *MIO10*, *AIM5*, or *AIM13*, the interaction of mitofilin/Fcj1 with the TOM complex is not disturbed but occurs with wild-type efficiency (von der Malsburg *et al.*, 2011), demonstrating that an intact MINOS complex is not required for the mitofilin/Fcj1–TOM interaction. Taken together, these data indicate two distinct forms of contact sites between outer membrane translocases and the inner

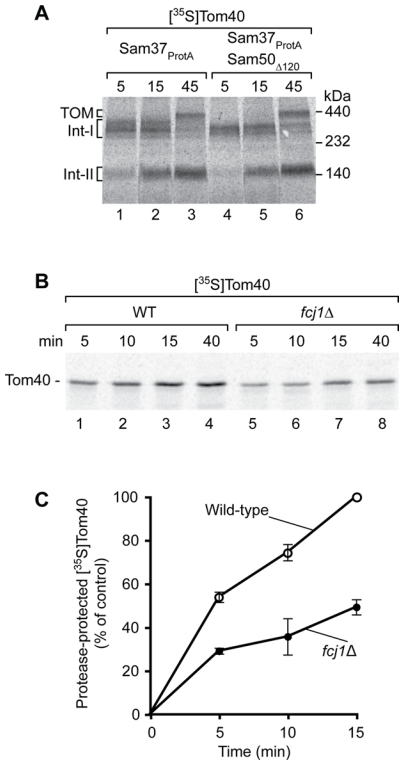

FIGURE 6: Mitofilin/Fcj1 is required at an early stage of Tom40 biogenesis. (A) [^{35}S]-labeled Tom40 was imported into Sam37$_{ProtA}$ and Sam37$_{ProtA}$ Sam50Δ_{120} mitochondria; this was followed by solubilization in digitonin buffer, blue native electrophoresis, and autoradiography. Int-I, precursor-SAM assembly intermediate I; Int-II, assembly intermediate II. (B) Radiolabeled Tom40 was incubated with wild-type (WT) and *fcj1*Δ mitochondria. Nonimported precursor was removed by proteinase K treatment, and mitochondria were subjected to SDS–PAGE and digital autoradiography. (C) Tom40 import experiments were performed as described in (B) and quantified. Data are represented as mean ± SE of the mean ($n = 3$), with the exception of the 10-min time point ($n = 2$; error bar represents range). The amount of protease-protected [^{35}S]Tom40 after 15-min import into wild-type mitochondria was set to 100% (control).

membrane organizing system are formed: a MINOS–SAM contact that requires the POTRA domain and likely includes the entire MINOS complex, and a mitofilin/Fcj1–TOM contact that does not depend on the other MINOS components.

We analyzed mitochondria that were isolated from yeast single-deletion mutants of each of the six MINOS genes for the biogenesis of outer membrane proteins. Remarkably, only mitochondria lacking mitofilin/Fcj1 were impaired in the assembly pathway of β-barrel proteins, as assessed with the model substrate Tom40. All other mutant mitochondria imported Tom40 with wild-type efficiency. Since *fcj1*Δ and *mio10*Δ mutants show a comparably strong degree of morphological alteration of the mitochondrial inner membrane (Harner *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012), it can be excluded that the β-barrel assembly defect is indirectly caused by the morphological defect. Moreover, we observed that the phospholipid profiles of *fcj1*Δ and *mio10*Δ mitochondria are similar to those of wild-type mitochondria. We dissected the biogenesis pathway of Tom40 into distinct stages and observed that the lack of mitofilin/Fcj1 affected an early import step that includes translocation of the Tom40 precursor through the TOM complex to the intermembrane space. The activity of the SAM complex itself was not compromised in *fcj1*Δ mitochondria, since the SAM-dependent precursor of Tom22 was efficiently assembled in the mutant mitochondria and the POTRA-dependent MINOS–SAM interaction was not required for Tom40 assembly.

These findings suggest a model in which mitofilin/Fcj1 is present in at least two pools. On the one hand, mitofilin/Fcj1 is a subunit of the MINOS complex. This complex is crucial for maintenance of inner membrane morphology and also mediates the interaction with the SAM complex. On the other hand, a fraction of mitofilin/Fcj1 molecules are also functional without the other MINOS components. These mitofilin/Fcj1 molecules interact with the TOM complex and are involved in the biogenesis of β-barrel proteins of the outer membrane. Interestingly, the import of intermembrane space proteins via the MIA pathway is only impaired in *fcj1*Δ mitochondria but not in deletion mutants of other MINOS components (von der Malsburg *et al.*, 2011), supporting the view that the roles of mitofilin/Fcj1 in promoting protein biogenesis via the β-barrel and MIA pathways are performed by the mitofilin/Fcj1 pool, which does not depend on an intact MINOS complex. It has been suggested MINOS, SAM, and TOM are part of a large endoplasmic reticulum–mitochondria organizing network (ERMIONE) that is involved in controlling mitochondrial architecture and biogenesis (van der Laan *et al.*, 2012). The findings reported here support the view that ERMIONE functions as a dynamic network (Zerbes *et al.*, 2012).

MATERIALS AND METHODS

Yeast strains

S. cerevisiae strains used in this study are derivatives of YPH499 (*MAT*a, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801*; Sikorski and Hieter, 1989). YPH499 strains *fcj1*Δ, *mio10*Δ, *mio27*Δ, *aim5*Δ, *aim13*Δ, and *aim37*Δ were generated by homologous recombination using *kanMX4* cassettes amplified from genomic DNA from strains *fcj1*Δ (BY4741), *mio10*Δ (BY4741), *mio27*Δ (BY4741), *aim5*Δ (BY4741), *aim13*Δ (BY4741), and *aim37*Δ (BY4741) obtained from Euroscarf (Frankfurt, Germany; Brachmann *et al.*, 1998). The strains Sam37$_{ProtA}$, Oxa1$_{ProtA}$, and Fcj1$_{ProtA}$ have been described previously (Kozjak *et al.*, 2003; Frazier *et al.*, 2006; von der Malsburg *et al.*, 2011). A strain expressing Mio27 fused to a C-terminal protein A tag for affinity chromatography was generated by homologous recombination using a cassette consisting of a TEV protease cleavage site, a protein A moiety, and a *HIS3* marker gene (Knop *et al.*, 1999). A similar cassette was transformed into Sam50$_{\Delta 120}$ cells (Kutik *et al.*, 2008) to generate the strain Fcj1$_{ProtA}$ Sam50$_{\Delta 120}$. A fragment encoding a *HIS3* marker gene, a *NOP1* promoter, a protein A moiety, and a TEV protease cleavage site was amplified from genomic DNA derived from Sam37$_{ProtA}$ cells and transformed into Sam50$_{\Delta 120}$ cells to generate the strain Sam37$_{ProtA}$ Sam50$_{\Delta 120}$. A cassette encoding a *kanMX6* module and a *GAL1* promoter was integrated 5′ of the *FCJ1* open reading frame by homologous recombination to generate strain Fcj1↓ (YPH499 *fcj1::kanMX6*, *PGAL1-FCJ1*; Longtine *et al.*, 1998).

Growth conditions, isolation of mitochondria, and analysis of protein content

For isolation of mitochondria, cells were grown at 30°C. Typically, cells were cultured in YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone, 3% [vol/vol] glycerol). For depletion of Fcj1, the strain Fcj1↓ (YPH499 *fcj1::kanMX6*, *PGAL1-FCJ1*) and the corresponding wild-type strain were precultured in YPGal medium

(1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone, 2% [wt/vol] galactose) for 6 h and transferred on YPG medium. After approximately three doubling times, 1% glucose was added to the medium to block expression of the *PGAL1-FCJ1* gene, and cells were harvested after 11 h. Mitochondria were isolated by sequential centrifugation as previously described (Meisinger *et al.*, 2006). Mitochondrial protein content was analyzed by SDS–PAGE and Western blotting. Alternatively, protein complexes were analyzed by solubilization in digitonin buffer (1% [wt/vol] digitonin, 20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol, 2 mM phenylmethylsulfonyl fluoride [PMSF]), blue native electrophoresis (Stojanovski *et al.*, 2007b), and Western blotting.

Protein import into isolated mitochondria

In vitro import reactions typically contained 50–80 µg mitochondria (protein amount) diluted in 100 µl import buffer (3% [wt/vol] bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine, 10 mM MOPS-KOH, pH 7.2, 4 mM ATP, 4 mM NADH, 5–10 mM creatine phosphate, 100–200 µg/ml creatine kinase; Ryan *et al.*, 2001; Stojanovski *et al.*, 2007b). Radiolabeled precursor proteins generated by in vitro translation in the presence of [^{35}S]methionine (TNT SP6 Quick Coupled kit; Promega, Madison, WI) were added to prewarmed import reactions (20°C/25°C). Samples were transferred on ice after different time points to terminate import reactions. Import of the precursors of ADP/ATP carrier, F_1-ATPase subunit β and cytochrome c_1 was terminated by addition of an AVO mix (8 µM antimycin A, 1 µM valinomycin, 20 µM oligomycin). Where indicated, nonimported precursor proteins were removed by incubation with 50 µg/ml proteinase K on ice for 15 min. Proteinase K was subsequently inactivated by addition of 2 mM PMSF. Mitochondria were washed with SEM buffer (250 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EDTA) and analyzed by SDS–PAGE or blue native electrophoresis, which was followed by digital autoradiography.

Preparation of yeast whole-cell extracts and affinity chromatography

Yeast cells were cultured in YPG medium at 30°C. Cells were harvested by centrifugation and washed twice with demineralized water and twice with washing buffer (20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol; Stroud *et al.*, 2011b; Zerbes *et al.*, 2012). Cells were frozen in liquid nitrogen and ground using a cryomill (20 min, 25 Hz). The resulting whole-cell powder was solubilized in solubilization buffer (20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol, 1% [wt/vol] digitonin, 2 mM PMSF, 1× EDTA free proteinase inhibitor [Roche, Indianapolis, IN], 30 µg/ml DNAse I); this was followed by a clarifying spin. Protein extracts were subsequently applied to IgG affinity chromatography. Unspecifically bound proteins were removed by extensive washing (20 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, 60 mM NaCl, 10% [vol/vol] glycerol, 0.3% [wt/vol] digitonin, 2 mM PMSF). Bound proteins were eluted by TEV protease cleavage, applied to SDS–PAGE, and visualized by Western blotting.

Phospholipid analysis

Isolated mitochondria were subjected to lipid extraction using chloroform/methanol (2:1; vol/vol) as previously described (Folch *et al.*, 1957). The organic phase was subsequently washed with 0.034% $MgCl_2$ solution (wt/vol), 2 N KCl/methanol (4:1; vol/vol) and methanol/water/chloroform (48:47:3; per vol). For separation of individual phospholipids, two-dimensional TLC using silica gel 60 plates (Merck, Darmstadt, Germany) was applied (first developing solvent: chloroform/methanol/25% NH_3 [68:35:5; per vol]; second developing solvent: chloroform/acetone/methanol/acetic acid/water [53:20:10:10:5; per vol]). After iodine vapor staining of TLC plates, phospholipids were scraped off and quantified according to Broekhuyse (1968).

Miscellaneous

For assessment of the mitochondrial membrane potential, the potential-sensitive dye dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) was used (Geissler *et al.*, 2000). For electron microscopy analysis, diaminobenzidine staining and imaging of cells were performed as previously described (von der Malsburg *et al.*, 2011; Zerbes *et al.*, 2012).

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